Reference: *Biol. Bull.* **164:** 124–135. (February, 1983)

α-LACTOSE BINDING HEMAGGLUTININS FROM THE ASCIDIAN *PHALLUSIA MAMILLATA* (CUV.)

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ABSTRACT

The carbohydrate binding specificities of *Phallusia mamillata* anti-rabbit and anti-human (A, B, O) hemagglutinins were determined by hemagglutinin inhibition tests. Both these molecules bind α-lactose, while only anti-HE hemagglutinins bind lactulose. In spite of this difference, absorption experiments did not show two distinct agglutinins for rabbit and human erythrocytes. Since both sugars are oligosaccharides which present a D-galacto-configuration, isolation by affinity chromatography with Sepharose was performed. The D-galactose-eluted fraction, investigated by PAGE and SDS-PAGE, appears to be the more anodal serum glycoprotein which consists of 2 subunits: the larger has a molecular weight of approximately 61–65,000 Daltons and the second one was 4000 Daltons smaller than the first. Gel filtration on Sephadex G 200 suggests that hemagglutinins represent a population of molecules in which the larger molecules (<200,000 Daltons) represent the greatest fraction.

INTRODUCTION

The presence of hemagglutinins has been demonstrated in worms, molluscs, arthropods, echinoderms and protochordates (Tyler and Metz, 1945; Tyler, 1946; Wright and Cooper, 1975; Parrinello and Patricolo, 1975; Amirante, 1976; Parrinello *et al.*, 1976). These molecules seem to be synthesized by hemocytes (Amirante, 1976) in the hemolymph; they can interact with foreign substances and show opsonic properties (Prowse and Tait, 1969; McKay and Jenkin, 1970; Cooper, 1976), forming a simple recognition system which enables invertebrates to discriminate between "self" and "non self". They are usually high molecular weight substances which appear to be protein made up of subunits forming large molecules which in no way resemble the immunoglobulins of the vertebrates (Tyler and Metz, 1945; Tyler and Scheer, 1945; Cohen, 1968; Acton *et al.*, 1969; Hall and Rowlands, 1974a; Pauley, 1974; Parrinello and Patricolo, 1975; Parrinello *et al.*, 1976). Their binding sites have specificity for carbohydrate moities, and several distinct subpopulations of active molecules can bind different sugars (Johnson, 1964; Sharon and Lis, 1972; Gold *et al.*, 1974; Hall and Rowlands, 1974b; Ryoyama, 1974; Anderson and Good, 1975; Hammarstrom *et al.*, 1977; Parrinello and Canicattì, 1982).

The sera of both lower and higher vertebrates also contain naturally occurring proteins which exhibit binding sites for carbohydrates (cf. Litman, 1976). These molecules can be immunoglubulins (Sela *et al.*, 1975; Karajalainen and Mäkelä, 1976) or non-immunoglobulin lectin-like proteins (Harisdangkul *et al.*, 1972; Litman, 1976). Since tunicates represent a phylogenetically critical taxon (Berril, 1955),

Received 8 July 1982; accepted 11 November 1982.

Abbreviations: PBS = phosphate buffered saline; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate; RE = rabbit erythrocytes; HE = human erythrocytes; HA-RE = erythrocyte-bound hemagglutinin.

studies of their sugar-specific agglutinins could contribute to an evaluation of the structural and functional evolution of these proteins.

The blood of ascidians contains hemagglutinins which react with vertebrate erythrocytes and bind the carbohydrate components on the cell surface (Anderson and Good, 1975; Wright and Cooper, 1975; Marchalonis and Warr, 1978; Parrinello and Canicattì, 1982). The agglutinin specificity has usually been determined by competitive binding studies involving the capability of monosaccharides or their derivatives to inhibit the agglutination of erythrocytes by agglutinin (Sharon and Lis, 1972). In a previous paper we showed that in the serum of *Ascidia malaca* two distinct agglutinins with similar sugar specificity ranges were responsible for rabbit and human erythrocyte agglutination (Parrinello and Canicattì, 1982). The specificity range includes saccharides containing a D-galacto-configuration (D-melibiose, D-raffinose, D-galactose, α-lactose, lactulose, L-arabinose) and allowed the isolation of the agglutinating serum fraction by biospecific affinity chromatography of the serum with Sepharose. *Phallusia mamillata* Cuv. possesses protein substances which agglutinate a variety of erythrocytes (Parrinello and Patricolo, 1975). The present report is concerned with the specificity of these hemagglutinins as determined by competitive binding studies and with the electrophoretic and immunological characterization of the isolated serum fraction.

MATERIALS AND METHODS

Phallusia mamillata were collected in the Gulf of Palermo. The blood, obtained by cardiac puncture, was centrifugated at 400 × *g*. The pooled serum, dialyzed with phosphate buffered saline at pH 7.4 (PBS), was divided into 2 ml aliquots and stored at −20°C.

Serial doubling dilutions of serum were prepared in U-bottom wells of microtiter plates (Cooke Engineering Co., Alexandria, Virginia) following the standard method (Kabat and Mayer, 1961). Hemagglutinating activity was determined with a suspension of washed erythrocytes (8×10^7 cells/ml). The agglutination trays were incubated for one h at 37°C and the assay read after 12 h at 4°C, the end titer being taken as the reciprocal of the last dilution giving a clear agglutination following gentle shaking.

The erythrocyte suspension (4×10^8 cells/ml, in PBS) was incubated with trypsin or bromelain (Sigma, 0.1 mg/ml) for 30 min at 37°C. The treated erythrocytes were washed 5 times with PBS.

Absorption studies were carried out as follows: packed erythrocytes (2 ml) and equal volumes of serum preparations were mixed and incubated at room temperature for 1 h, and at 4°C for 12 h, with occasional shaking. The absorbed serum was obtained by centrifugation of the mixture.

Hemagglutination inhibition was tested by the same method used for *A. malaca* agglutinins (Parrinello and Canicattì, 1982). Dialyzed serum (0.25 ml) was added to 0.05 ml of a 2-fold serial dilution of carbohydrates using microtiter plates. After a 30 min incubation at 37°C, 0.025 ml of erythrocyte suspension (8×10^7 cells/ml) were added. The hemagglutination test was carried out under the above mentioned conditions and the last sugar dilution capable of inhibiting hemagglutination was evaluated.

Protein content was determined by the Folin-Ciocalteau method (Lowry *et al.*, 1951). Bovine serum albumin was the reference standard. Each value was expressed as average of three determinations ± SE.

Gel filtration experiments were performed using a 1 × 60 cm Sephadex G-200 column equilibrated with PBS. Serum samples were concentrated by ultrafiltration in a Diaflo equipped with a UM 2 membrane (Amicon Corp., Lexington, MA) and then dialyzed overnight against the starting buffer. One ml of three-fold concentrated serum was loaded onto the column at 20°C. At a flow rate of 8 ml per h, 0.5 ml fractions were collected, read for UV (280 nm) absorbancy, and tested for hemagglutinating activity. Dextran blue 2000 (Pharmacia), human IgG, bovine serum albumin and cytochrome C (Sigma) were used to calibrate the column (Andrews, 1965).

Affinity chromatography column (1.5 × 15 cm) were filled with acid-treated Sepharose CL-6B (Pharmacia) prepared according to Ersson *et al.* (1973). The gel suspended in 0.2 M HCl was stirred at 50°C for 3 hours and after several washings, equilibrated with PBS. After a 20 ml load of serum the columns were washed with 10–12 bed volumes of NaCl (1 *M*) and then eluted with 0.2 *M* sugar in NaCl (1 *M*). The sugar-eluted fractions, dialyzed against NaCl (1 *M*) and PBS to remove the sugar, and the unspecific eluted fractions were assayed for hemagglutination activity with rabbit (RE) and human A, B, O erythrocytes (HE).

Electrophoresis in 7.5% polyacrylamide gels (PAGE) was performed as described by Davis (1964). The sample (50 μl) was added to an equal volume of 5 m*M* Tris-glycine, pH 8.3, containing 40% sucrose and the mixture was deposited on the spacer gel. A constant current of 1 mA/tube was supplied until the buffer line passed through the spacer gel into the small-pore gel. The current was then increased to 3 mA/tube and the electrophoretic run was carried out for 60 min. Proteins were stained for 1 h with Coomassie Blue. Following electrophoresis, color reactions of the gels were performed to investigate the glycoprotein or lipoprotein nature of the isolated active fraction. Periodic acid-Schiff's reagent (Felgenhauer, 1970) and Sudan Black staining (Prat *et al.*, 1969) were used as described by Maurer (1971).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Shapiro *et al.*, 1967; Weber and Osborne, 1969) was carried out as described by Gavery *et al.* (1977). Gels containing 5.6% acrylamide and 0.2% *N*,N-methylenbisacrylamide were used. The samples were dialyzed against 0.02 *M* Tris-acetate buffer containing 2% β-mercaptoethanol and 2% SDS and then heated for ten min at 100°C. Phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (Bio-Rad) were the molecular weight protein standards for SDS gel electrophoresis. The electrophoretic run was carried out for 4 h at 4 mA/tube.

Antiserum to rabbit erythrocyte-bound hemagglutinins was produced preparing the antigen according to Amirante (1976). *P. mamillata* serum was absorbed with RE as previously described. The agglutinated erythrocyte mass (HA-RE), obtained by centrifugation of the mixture, was washed several times with PBS to remove serum components. The rabbit which had supplied the erythrocytes for absorption was injected as follows: 5 ml of HA-RE emulsified with an equal volume of Difco Freund's complete adjuvant divided in small doses was injected intramuscularly into both hind legs; a second intramuscular injection identical to the first was given on day 30; subcutaneous injection of 5 ml of HA-RE in PBS was given in two sites on day 60. The animal was bled from an ear vein on day 40 and by cardiac puncture on day 70. The sera were not pooled but kept in separate 2 ml lots and stored at −20°C.

Microimmunoelectrophoresis (Scheidegger, 1955; Ouchterlony and Nilsson, 1973) was carried out using 1.5% agarose (Bio-Rad) and Trisbarbital sodium barbital buffer, pH 8.8. A current of 4 mA/strip was applied for 50 min. Antiserum diffusion was allowed to occur in a moist chamber at room temperature generally for 24 to 72 h. Dried agarose films were stained with Coomassie Blue.

TABLE I

Hemagglutinating activity of Phallusia mamillata *serum against untreated and protease-treated erythrocytes*

Treatment[1]	Hemagglutinating titer[2] (reciprocal) Rabbit Erythrocytes	Hemagglutinating titer[2] (reciprocal) Human Erythrocytes (A, B, O)
—	32–128	4–16
Trypsin	254–2048	32–128
Bromelain	254–2048	254–2048

[1] Erythrocytes incubated with enzyme (0.1 mg/ml) for 30 min at 37°C.

[2] Hemagglutination titer ranges obtained from repeated tests and expressed as reciprocal of the last serum dilution giving agglutination.

RESULTS

Hemagglutinating activity of the serum against untreated and treated erythrocytes. Absorption experiments

The *P. mamillata* serum showed a high agglutinating activity against both rabbit and human A, B, O erythrocytes. As shown in Table I, the hemagglutination titers of the anti-RE (32–128) were higher than those of the anti-HE (4–16). The protease treatments enhanced the susceptibility of erythrocytes to agglutination: bromelain was more effective than trypsin in increasing the susceptibility of RE and HE to agglutination by *P. mamillata* agglutinins.

To verify the occurrence of specific hemagglutinins, the absorption experiments were performed. Sera showed no residual activity when absorbed with either RE or HE (A, B, O groups).

Sugar specificity of the anti-RE and anti-HE agglutinins

Carbohydrate binding specificity of *P. mamillata* anti-RE agglutinin was examined using various monosaccharides, oligosaccharides and modified sugars to inhibit hemagglutination of the untreated erythrocytes. The experiments showed that the specificity of the agglutinins is limited to α-lactose which inhibits the agglutination reaction up to final concentration of 71.5 μ moles/ml, SE ± 14 (Table II). No significant inhibition was observed with the monosaccharides D-mannose, D-glucose, D-fructose, L-sorbose, D-ribose and D-xylose; the oligosaccharides D-melezitose, maltose, D-cellobiose, D-trehalose, D-melibiose, and D-raffinose and lactulose; the modified sugars D-digitoxose, 2-deoxyglucose, 2-deoxyribose, salicin, sialic acid, N-acetyl-D-mannosamine, lactobionic acid and the two p-nitrophenyl-(α or β)-D-galactosides, when tested at final concentrations (0.4 M) higher than necessary to obtain complete inhibition with α-lactose. The polyalcohols D-mannitol, D-sorbitol, myo-inositol and dulcitol were also inactive.

Each of the above listed sugars were also tested for inhibition of agglutinating capability of the serum with human A, B, O erythrocytes. It was found that α-lactose inhibits the agglutination reaction at a lower concentration than that required to inhibit anti-RE agglutination. Lactulose interacts weakly with anti-HE agglutinins as a high concentration was required to inhibit agglutination (Table II).

Divalent cations are not necessary for carbohydrate-agglutinin binding as shown by the results of the inhibition experiments in which reaction mixtures containing

TABLE II

α-Lactose and lactulose inhibition of the agglutination of rabbit and human erythrocytes with Phallusia mamillata *serum preparations.*

Sugar	Minimum sugar concentration (μ mole/ml) for inhibition[1]			
	Rabbit erythrocytes	Human erythrocyte		
		A	B	O
α-lactose	71.5 ± 14.4	50.5 ± 17.8	28.7 ± 10.7	25.0 ± 10.5
lactulose	—	167.4 ± 33.0	144.0 ± 56.8	147.7 ± 54.3

[1] Average values ± SE obtained by testing erythrocytes from 7 specimens.
— = sugar ineffective at maximum concentration (0.4 *M*) used for inhibition.

EDTA (0.01–0.1 *M* final concentration, pH corrected to 7.4) were tested with HE and RE.

Sugar-agglutinin binding is a reversible process. Serum preparations incubated with α-lactose at the maximum concentration used in this study show agglutinating capability after exhaustive dialysis with saline solution.

Gel column chromatography of the serum

The protein content determinations of serum samples concentrated by ultrafiltration have shown that the hemagglutinating titer of the *P. mamillata* serum is related to the protein concentration: 167 ± 1.5 μg protein per ml produces a hemagglutinating titer of 16–32; ten-fold concentrated serum (525 ± 1.8 μg protein/ml) gave a titer of 128–256.

To determine the relative size of the molecules responsible for the hemagglutinating activity, gel filtration of the concentrated serum (525 ± μg protein/ml) on a calibrated Sephadex G-200 column (1 × 60 cm) was performed. Figure 1a shows a typical protein elution profile in which the agglutinating activity against RE and HE appeared in the single major protein fraction eluted with the blue dextran. Figure 1b shows the column chromatography separation of serum previously frozen for 8–12 months. Three separate peaks were obtained; hemagglutinating activity was mainly associated with the major protein fraction which was eluted at 13.5 ml with the blue dextran, however hemagglutinins were also found in the fractions eluted at volumes ranging between 15 and 25 ml which include the elution volumes of human IgG (160,000 Daltons) and ovalbumin (43,000 Daltons). This suggests that the hemagglutinins in a long-time frozen and concentrated serum were associated with molecules heterogeneous in size.

Affinity chromatography of the agglutinins

The demonstration of the reactivity of *P. mamillata* anti-RE and anti-HE agglutinins with oligosaccharides containing D-galactose (α-lactose and lactulose) and the availability of D-galactose support (Sepharose, Pharmacia) led us to explore the use of this medium for purification of the agglutinins. In a typical separation experiment, anti-RE and anti-HE agglutinins were isolated from 20 ml of whole serum (protein content 358 ± 1.1 μg per ml) on a column of cross-linked acid-treated Sepharose 6B (Fig. 2). After this treatment a greater number of end groups became available for reaction with agglutinins (Ersson *et al.*, 1973), which are adsorbed by

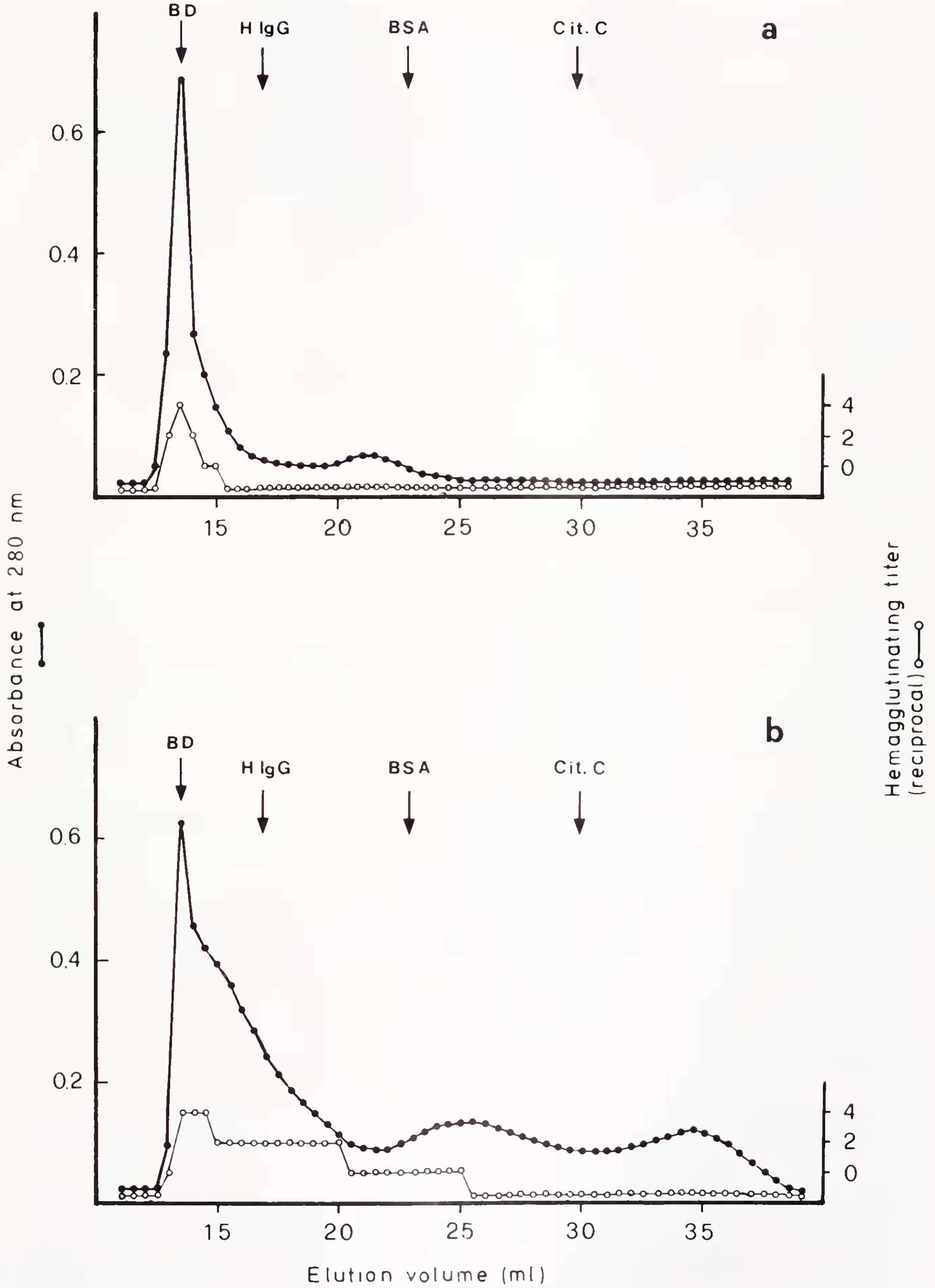

FIGURE 1. Sephadex G-200 chromatography of *Phallusia mamillata* concentrated serum (protein content ~1 mg/ml). The column (1 × 60 cm) was equilibrated and eluted with PBS. The flow rate was 8 ml/h, 0.5 ml fractions were collected. The elution positions of Blue Dextran 2000 (BD), human IgG (HIgG), bovine serum albumin (BSA), and citochrome C (Cit. C) are indicated. The hemagglutinating titers, expressed as reciprocal of the serum dilution, were obtained using RE and HE (A, B, O). In "b" the elution profile of serum frozen for 8–12 months is shown.

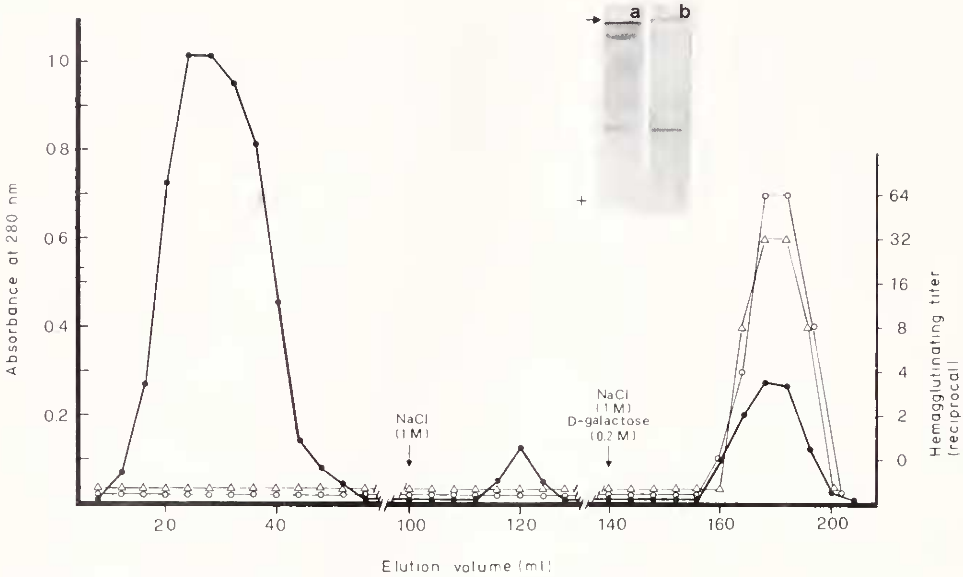

FIGURE 2. Affinity chromatography of *Phallusia mamillata* hemagglutinins on acid-treated Sepharose CL-6B. PBS-dialyzed serum (20 ml) was applied to a column (1.5 × 15 cm) at 25°C. The column was washed with 1 *M* NaCl and, at the point indicated, elution was started with 1 *M* NaCl plus 0.2 *M* D-galactose. The flow rate was 20 ml/h. The eluted fractions were dialyzed against NaCl (1 *M*) and PBS to remove the sugar. All the fractions were assayed for hemagglutinating activity with RE and HE, hemagglutinating titers were expressed as reciprocal of the serum dilution. Inset: developed portions of the polyacrylamide gels showing electrophoretic patterns; a) whole serum, b) pooled D-galactose eluted fraction showing maximum hemagglutinating titer. Arrow indicates the origin.

the gel bed. The first peak observed in the elution profile contains all the inactive proteins. The column was then washed with 1 *M* NaCl to elute the non-agglutinating fractions from Sepharose. Finally, when the eluate had reached a constant low absorbance at 280 nm, anti-RE and anti-HE agglutinins were displaced from the Sepharose bed by a solution of D-galactose (0.2 *M*) in 1 *M* NaCl.

These experiments clearly demonstrate that 0.2 *M* D-glucose in 1 *M* NaCl solution, a non-inhibitor of the agglutinin-polysaccharide interaction, did not elute agglutinins specifically bound to Sepharose, whereas α-lactose, like D-galactose, (0.2 *M* solution containing 1 *M* NaCl) readily displaced them from the gel bed. NaCl solution (1 *M*) or sugar in PBS (0.2 *M* solution) were unable to elute the agglutinins.

Electrophoretic and immunological characterization of the isolated agglutinins

The whole serum and the D-galactose-eluted protein were investigated by polyacrylamide gel electrophoresis. In Figure 2 the patterns of the *P. mamillata* serum (Fig. 2a) and the isolated fraction (Fig. 2b) are compared; Coomassie blue staining revealed that the agglutinating activity was principally connected with the large, more anodal protein component of the serum. Color reaction of the gels with periodic acid-Schiff's reagent after electrophoresis showed the glycoprotein nature of this serum component.

To study the D-galactose-eluted fraction by immunological methods, antiserum against the agglutinin bound to RE was produced. The immunoelectrophoresis results presented in Figure 3 show that the anti-serum reacts with two distinct com-

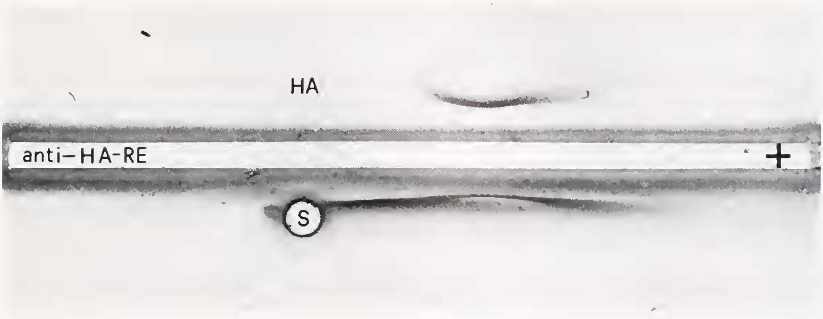

FIGURE 3. Immunoelectrophoresis in which antiserum, produced against rabbit erythrocyte-bound agglutinins (anti-HA-RE), reacted with D-galactose-binding hemagglutinin isolated by Sepharose affinity chromatography (HA) and normal *Phallusia mamillata* serum (S). The fractions used were those resulting in the maximum hemagglutinating titer.

ponents of the whole serum, whereas only the more anodal immunoprecipitates can be observed as a result of interaction between the antiserum and the D-galactose-eluted fraction. The morphology of the latter arc suggests that it represents several immunologically distinguishable components.

Molecular weight determination of the reduced agglutinins

In electrophoresis on SDS-polyacrylamide gel, the electrophoretic mobility of each protein chain is inversely proportional to the logarithm of its molecular weight (Shapiro *et al.*, 1967; Weber and Osborne, 1969). When D-galactose-eluted agglutinin preparations were reduced and denaturated by SDS, polyacrylamide gel electrophoresis showed that these proteins were dissociated into two subunits with similar molecular weights (Fig. 4a) determined from standard curves of the type shown in Figure 4b. The values were obtained from six independent experiments. The heavy chain ranged from 61,000–65,000 Daltons and the other subunit, in each determination, was always about 4000 Daltons lighter (mean values: 64,000 and 60,000 ± 1630 SD). Very low concentrations of low molecular weight protein chains ($21–23 \times 10^3$ and $35–40 \times 10^3$ Daltons) can occasionally be found, but they could be a result of the denaturating experimental conditions.

DISCUSSION

We have shown that naturally occurring hemagglutinins from *Phallusia mamillata* react with the sugar moities of human and rabbit erythrocyte membrane receptors. The inhibition data suggest that the anti-RE agglutinin presents a binding site which reversibly interacts with the α-lactose. The whole of this sugar molecule seems to be involved in the binding, in fact neither D-galactose nor D-glucose by themselves, nor other oligosaccharides containing these monosaccharides, are capable of inhibition. A D-galacto-configuration is also involved in the binding of the agglutinin with the HE: both α-lactose and lactulose (4-(β-D-galactosyl)-D-fructose) inhibit this agglutination. In spite of this specific binding of lactulose and of the different inhibition power of α-lactose, two different agglutinins for RE and HE could not be demonstrated by absorption experiments. These apparently contradictory results could be explained by the hypothesis that the variation in the inhibition powers of these sugars is caused by some slight difference in the binding sites

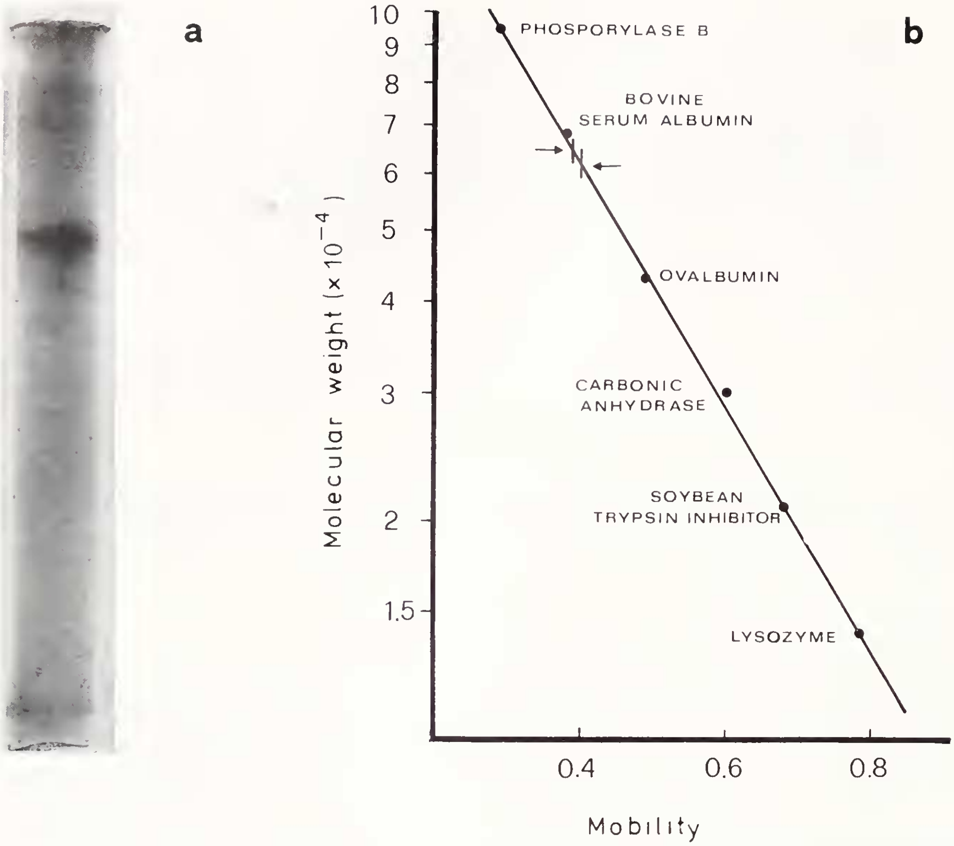

FIGURE 4. (a) SDS-polyacrylamide gel electrophoresis of *Phallusia mamillata* D-galactose-binding hemagglutinins isolated by Sepharose affinity chromatography. The eluted fractions used were those resulting in the maximum hemagglutinating titer. (b) Plot of molecular weight ($\times 10^{-4}$) against electrophoretic mobilities of different protein chains on SDS-polyacrylamide gel electrophoresis. The mobility was calculated according to Weber and Osborne (1969). The vertical bars indicate the estimated standard deviations of *Phallusia mamillata* hemagglutinin subunits (arrows) molecular weights.

of the hemagglutinins. D-galactose, while not involving divalent cations, seems principally responsible for the receptor-agglutinin interaction: agglutinin can bind to the end D-galactose groups of the acid-treated Sepharose and D-galactose solution can elute it; D-glucose was unable to elute the agglutinin even when in highly concentrated NaCl solution. A galactosyl group was apparently connected with recognition very early in phylogeny. D-galactose reactivity characterizes plant and invertebrate agglutinins from several sources (Johnson, 1964; Ersson *et al.*, 1973; Gold *et al.*, 1974; Ryoyama, 1974; Parrinello and Canicattì, 1982) and it could be a common character of a primordial self-nonself recognition mechanism. The particular spatial configuration of the galacto-pyranosyl structure makes this a prominent group which binds agglutinin even if it is contained in masked erythrocyte surface receptors, as suggested by the increased agglutinability of the protease-treated HE and RE.

Regarding the binding site, affinities exist between *P. mamillata* and *Ascidia malaca* hemagglutinins. Analysis of anti-RE and anti-HE *A. malaca* agglutinins (Parrinello and Canicattì, 1982) has suggested that the size of the combining site corresponds to a disaccharide with a specificity for saccharides containing a D-galacto-configuration. The differences involve the specificity range of *A. malaca* agglutinins which includes D-galactose, L-arabinose, D-melibiose, D-raffinose, α-lactose and lactulose.

A most convenient method which fulfills the requirements of isolation of agglutinins in highly purified form is affinity chromatography, which is based on the ability of agglutinins to bind carbohydrates specifically and reversibly. We have already reported the specific binding of *A. malaca* agglutinins to Sepharose gel and their elution by specific sugar (Parrinello and Canicattì, 1982). The *P. mamillata* anti-RE and anti-HE agglutinins were isolated by this method too, but a sugar solution of a high ionic strength was required to break the sugar-protein interactions. In this respect these agglutinins differ from those of *A. malaca* which were eluted by a D-galactose solution in PBS.

The isolated agglutinins seem to correspond primarily to the most anodal protein fraction of the serum when analyzed by polyacrylamide gel electrophoresis. The Schiff's reagent demonstrated the glycoprotein nature of this fraction. In immunoelectrophoresis it appears as a single electrophoretic band when tested with an antiserum versus erythrocyte-bound agglutinins, however the morphology of the immuno-precipitate suggest that it can be formed of several antigens. The ability of this antiserum to precipitate two different components from the whole serum could depend on the antigen preparation used for immunization or it could be due to cross reaction.

Molecular sieve column chromatography of the serum on Sephadex G-200 beads indicates that hemagglutinins can be large molecules with molecular weights greater than 200,000 Daltons. Most of the agglutinating activity appeared in the major protein fraction eluted with blue dextran. As shown by gel filtration experiments, freezing may alter the hemagglutinin enough to produce smaller agglutinating molecules. The elution profile of the frozen serum showed a smaller peak of activity a few tubes behind the blue dextran suggesting hemagglutinin size smaller than 160,000 Daltons; activity could also be found in the fractions containing proteins with molecular sizes about that of ovalbumin (43,000 Daltons). These findings suggest that the agglutinating molecules can be composed by subunits which associate to form macromolecular complexes.

Several papers indicate that invertebrate agglutinating sugar-specific proteins are different from agglutinating antibodies that vertebrates possess. Recently Marchalonis and Warr (1978) suggested that the subunits of the naturally occurring DNP-binding protein of the ascidian *Pyura stolonifera* resembles heavy chains (μ) of mammalian IgM when studied by PAGE analysis, in which their molecular weights appear to be $65–70 \times 10^3$ Daltons. We found that the subunits of the isolated sugar-specific agglutinins of *P. mamillata* are similar in size to those of *Pyura* and show medium molecular weight of 60,000 and 64,000. In this respect the ascidian Sepharose-isolated hemagglutinins could be a starting point for the study of the evolution of these molecules. Discussion of such a process will be possible when data on amino acid composition, carbohydrate content, and structural molecular organization can be evaluated.

ACKNOWLEDGMENT

We would like to express our appreciation to Prof. M. Ruccia, Institute of Organic Chemistry, University of Palermo, for his critical reading of the manuscript.

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